COLICIN-TOLERANT MUTANTS: AN APPROACH TO THE GENETICS OF MEMBRANES*

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* Supported by grants from the National Institutes of Health (AI 03038) and the National Science Foundation (GB-5304X).

Since our chairman has allotted to me more time than is required for presenting my experimental results I wish to devote a few minutes to some comments of a historical-methodological nature that may be of value in the framework of the present conference.

The study of biological membranes appears to me (hence my own interest in it) to be the key to supramolecular biology, that is, to the study of the arrangement of different molecular species that form the operational framework of the living cell, in the same way as molecular biology is the study of the macromolecules that are the functional elements of the cellular machinery.

The essentials of molecular biology are, first, the clarification of the linear organization of information in the genetic molecules, the nucleic acids, and their primary derivatives, the proteins; second, the study of the conversion of the linear informational molecules into their functionally active forms, the folded and internally bonded protein and nucleic acid molecules; and third, the analysis of the regulatory mechanisms that control the function of these molecules -- the induction and repression of genes and the allosteric activation or inhibition of enzymes. In the cell, however, the macromolecules must be arranged in specific patterns of high functional significance. For example, the biosynthesis of some complex carbohydrates in the bacterial envelope depends on coordinate function of many enzymes, which, like workers on an assembly line, construct macromolecules from subunits through subassemblies attached to lipidic carriers (Robbins et al., 1966). Likewise, oxidative phosphorylation and photophosphorylation in chloroplasts are in all probability dependent on structuralization of active elements into

precise functional assemblies. It is presumably the role of membranes to provide the framework for such organized assembly of enzymes and carriers.

To the molecular biologist the central problems of supramolecular biology appear to be two, one functional and one genetic:

1. Does the organizational framework in membrane and other cellular structures contribute to functional regulation? Is the function of enzymes, and possibly also of nucleic acids, regulated at least in part by their location next to other specific components?

2. Is the arrangement of components in the framework determined mainly by the properties of the individual components (in the same way as the tertiary structure of a protein molecule, and even the quaternary structure of certain enzyme complexes, are determined by the primary amino acid sequence), or is there some source of organizational information besides the intrinsic properties of the components themselves? And if so, does this additional information reside in the biosynthetic history of the components themselves (as might be the case for a group of proteins coded by successive segments of a polycistronic messenger RNA), or is it of the "primer" kind, in the sense that the preexisting framework determines the specific location and mutual arrangement of proteins and other molecules synthesized severally and independently?

The last question deals with a fundamental problem of cellular heredity, the role of preexistent structure in cellular morphogenesis. This problem has been discussed by Sonneborn in this Symposium. I wish to pose only the following question, of direct concern to membrane workers: is there any reliable evidence that a

typical membrane structure can be initiated de novo rather than under the priming influence of a preexisting membrane? Findings with certain animal viruses (see Dales, contribution to this Symposium) suggest that this may be so, but direct evidence on truly cellular membranes would be highly desirable.

If "membranology" is indeed a branch of supramolecular biology as defined above, it may pay us to consider the specific methodological roots of molecular biology in order to seek possible approaches to our tasks. The early paths of molecular biology, in addition to the general progress of biochemistry, have been two: the study of molecular structure, mainly by x-ray diffraction, and the study of microbial genetics. The greatest triumphs of the first path have been the invention of the α-helix, the discovery of the double helical structure of DNA, and more recently the detailed resolution of the structure of some proteins such as myoglobin, hemoglobin, and lysozyme. The second path has contributed the identification of DNA and viral RNA as genetic materials, the clarification of the fine structure of the gene, and the analysis of regulation of gene function. It is on the firm basis posed by these main approaches that the more recent advances of molecular biology, such as the deciphering of the genetic code, have developed. A remarkable feature of molecular biology is that the studies of molecular structure and of microbial genetics, although occasionally convergent, did proceed mainly in mutual isolation. Paradoxical as it may sound, this isolation may have been beneficial. What I mean is that, in the early stages of an experimental approach, a preoccupation with observations from a completely different approach can often interfere with the internal logical development of a field

of research. Where would cellular genetics be today if it had tried to search for mutants with altered molecular structure of proteins or DNA? Or, for people working on the molecular structure of tobacco mosaic virus, what use would have been worrying about the viral mutants that proved so useful in deciphering the genetic code?

What I am driving at is that at least for some time the study of membranes by genetic means, which I am hopefully advocating, may well have to bypass, if not ignore, much of the elegant structural and physicochemical work on natural and artificial membranes. An interesting illustration of this is the progress being made, by purely genetic and biochemical means, in the isolation and identification of two important functional constituents of the bacterial cell envelope, the β-galactoside "permease" protein of Escherichia coli (Kennedy, 1966) and the lipid carrier for peptidoglycan and O-antigen biosynthesis in bacteria (Wright et al., 1967; Higashi et al., 1967). Neither of these substances could have been detected, let alone identified, by structural or analytical studies alone, because they turned out to be very minor components, quantitatively, of the protein and lipid fractions of the bacterial envelopes.

What routes are membrane geneticists to follow? Here again molecular biology may help show the way. The genetic approach has two alternatives. One is to trust in accidental genetic observations, such as pneumococcal transformation, which led to the identification of DNA as the genetic substance of bacteria (Avery et al., 1944), or coordinate derepression and polar mutation, which led to the concept of the multi-gene operon (Jacob and Monod, 1961). The other alternative is

to define a specific cellular function or group of functions and then find a set of genetic changes that alter them; thereby one can dissect the underlying mechanism into individual biochemical steps. The best example of this rational approach is the analysis of cellular functions by conditional lethal mutants of microorganisms: nutritional mutants of fungi and bacteria (Beadle, 1945) or morphogenetic mutants of bacteriophage (Epstein et al., 1963). A limitation of this approach is, for the time being, its almost complete restriction to micro-organisms.

The remaining part of my talk is the presentation of one example of genetic approach to membrane problems in bacteria. I should point out that this is not the only example; others, especially Jacob and his coworkers (1966), are studying conditional lethal mutants of bacteria that exhibit alterations in membrane structure and function.

The approach presented here consists of a study of the mode of action of colicins, combined with the use of bacterial mutants altered in their response to these agents. Colicins are protein antibiotics produced by specific enteric bacteria, including many strains of Escherichia coli, and lethal for other bacterial strains (Fredericq, 1957; Nomura, 1963). The main common properties of colicins, including those relevant to the present discussion, are listed in Table 1. The essential ones are (1) that colicins act from sites accessible on the bacterial surface since they can be removed by tryptic digestion; (2) that the lethal effects are often repaired by such enzymatic removal, hence must be reversible damages; (3) that for most colicins the action follows one-hit kinetics as a function

of the amount of colicin, indicating the existence of amplifying mechanisms which generalize the effects of a single colicin molecule; and (4) that each colicinogenic strain is immune to the colicin it produces, an indication that the ability to produce colicin is accompanied by an alteration of some cellular surface components such that it prevents the response to colicin. The colicin molecule itself may be a membrane constituent, possibly related to substances mediating sexual conjugation in Escherichia coli (see Hayes, 1964).

The known colicins can be classified by at least two criteria: by their modes of action and by the patterns of bacterial mutations that produce loss of surface receptors for colicins (hence inability to adsorb these colicins and complete resistance to them). Table 2 presents as a matrix the classification of a selected group of different colicins by these two criteria. It is clear that the two classifications do not coincide. Hence, following Nomura (1967), we can provisionally postulate, on the one hand, a set of receptors for certain groups of colicins and, on the other hand, a set of biochemical "targets," each representing the primary functional site on which the inhibitory action of certain colicins is exerted. This situation is schematically represented in Figure 1. Each of the receptors is represented as able to provide their colicins an access to a subset of biochemical targets, and each target is represented as responding to colicins acting from a subset of receptors.

This scheme is obviously a provisional one; the most important questions remain to be answered. What are the hypothetical biochemical targets which control the course of energy metabolism, or the effects on cellular DNA, or on the ribosomal properties? Where are they located

in the bacterial envelope? What membrane constituents are involved: phospholipids, proteins? Does a colicin affect its targets by acting itself as an enzyme from its receptor site, or by activating enzymes located in the cell membrane? Or is the amplification, characteristic of colicin action, due to some spreading effect along the cell membrane? Could such a spreading effect be mediated by a two-dimensional wave of allosteric changes of state (Changeux et al., 1967) in some membrane proteins?

Even though all these questions remain to be answered, the scheme outlined in Figure 1 provides a point of departure for a genetic analysis. We can ask: are there bacterial mutations that produce resistance to colicins by affecting the targets rather than the surface receptors? Such mutations might be found by selecting bacterial mutants resistant to two colicins, such as E1 and K, that have different receptors but apparently similar targets.

A search for such mutants has led, not to the discovery of some types of target mutants, but rather to the isolation of an unexpected class of mutants, called tolerant (= tol), which have normal receptors (since they adsorb colicins normally) but fail to respond to specific groups of colicins. The tolerance patterns of the mutants studied in our laboratory (Nagel de Zwaig and Luria, 1967) are shown in Table 2; similar and some additional patterns have been found by Nomura and Witten (1967). The significant finding is that the way in which colicins can be grouped on the basis of the patterns of joint tolerance in these mutants coincides neither with the grouping by common receptors nor with that by common targets. This indicates an additional complexity in the scheme shown in Figure 1: we must

invoke formally a system of specific interactions between certain subsets of receptor-absorbed colicins and certain subsets of targets, interactions which are subject to specific alterations by the various tol mutations.

A study of the physiological properties of tol mutants (Nagel de Zwaig and Luria, 1967) has provided some significant indications that the cellular envelopes in these mutants are structurally altered. Thus the tol II and tol III mutations, which map in two different genes very near the gal region in the E. coli chromosome, cause an increased sensitivity to deoxycholate and to EDTA and, more interesting, a marked fragility of the bacterial cells, which causes them to break up and die spontaneously during growth. This happens even in hypertonic sucrose media such as are known to protect bacterial spheroplasts against lysis.

Even more interesting are the mutants of the tol VIII class: they are extremely sensitive to deoxycholate (not to EDTA) and are apparently freely permeable to methylene blue, acridine dyes, and other organic cations, which therefore kill the mutant cells at concentrations that are completely innocuous to the parental bacteria.

This set of observations is quite intriguing. It suggests that the postulated interactions between the colicins and their biochemical targets (see Figure 1) are mediated through a set of chemical cell constituents which are subject to mutational changes and which play essential roles in maintaining the integrity of the cellular envelope, presumably the cytoplasmic membrane, and in determining its permeability properties. Hence, we think that the study of colicin-tolerant mutants

has provided a method that can serve for the isolation of all sorts of membrane mutants. The functional and biochemical analysis of these mutants should throw light on a number of functional components of the membrane.

Some observations by Nomura and Witten (1967) are also encouraging: some classes of tol mutations are temperature dependent, in the sense that the mutant bacteria are sensitive to colicins at 30° but become tolerant after a brief treatment at 42° even in the absence of growth. This suggests that this tolerance results from the inactivation of some cellular components, presumably proteins. At least one of these heat sensitive components appears to be essential for the bacterial cell, because one class of temperature-dependent tol mutants cannot grow at the temperature that converts the cells to colicin-resistance. Hence, there is at least some indication that the approach through tolerant mutants may serve to reveal and identify essential components of the bacterial membrane which regulate some of the vital steps of cellular metabolism.

I must emphasize that the observations summarized above are only a hopeful beginning of a potentially useful approach. They are presented mainly as an example of the way the microbial geneticist operates. It is to be hoped, however, that the ultimate results of this approach will bring out some important features of the functional organization of bacterial membranes that can be of interest to all membranologists.

Table 1

Classification of a group of colicins

Mode of action[a] (Biochemical target)	Common cellular receptors[b]			
1. Interference with energy metabolism	A	E1	K	Ib
2. DNA damage		E2		
3. Ribosomal damage		E3		

[a] The colicins in each row have similar killing action on susceptible bacteria.

[b] The colicins in each column are those whose attachment sites on the bacterial cell wall can be lost by a single bacterial mutation.

Table 2

Mutations to colicin-tolerance in *E. coli* K-12

Mutant class	Tolerant to colicins	Sensitive to colicins
tol II	A, E1, E2, E3, K	I, B, V
tol III	A, E2, E3, K	E1, I, B, V
tol VIII	E1	E2, E3, K, I, B, V

LEGEND FOR FIGURE

Figure 1. Relation between colicin receptors and biochemical targets in the bacterial cell envelope.

The boxes labeled R_A, R_E ... represent the receptors for colicins of groups A, E, ... The boxes labeled T1, T2, ... correspond to the biochemical targets of colicin action (see Table 1). The extent to which different receptors may overlap structurally is unknown.

The arrows represent the formal interconnections between receptors and targets deduced from the information in Table 1. The tol mutations listed in Table 2 uncouple various sets of interconnections. (o : blocked by tol II mutation; x : blocked by tol III mutation; • : blocked by tol VIII mutation).

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